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Gelatin functionalised porous titanium alloy implants for orthopaedic applications

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ABSTRACT

In the present work, we studied the immobilisation of the biopolymer gelatin onto the surface of three dimensional (3D) regular Ti6Al4V porous implants to improve their surface bio-activity. The successful immobilisation of the gelatin coating was made possible by a polydopamine interlayer, a polymer coating inspired by the adhesive nature of mussels. The presence of both coatings was first optimised on two dimensional titanium (2D Ti) substrates and confirmed by different techniques including X-ray photelectron spectroscopy, contact angle measurements, atomic force microscopy and fluorescence microscopy. Results showed homogeneous coatings that are stable for at least 24 h in phosphate buffer at 37 °C. In a next step, the coating procedure was successfully transferred to 3D Ti6Al4V porous implants, which indicates the versatility of the applied coating procedure with regard to complex surface morphologies. Furthermore, the bio-activity of these stable gelatin coatings was enhanced by applying a third and final coating using the cell-attractive protein fibronectin. The reproducible immobilisation process allowed for a controlled biomolecule presentation to the surrounding tissue. This newly developed coating procedure outperformed the previously reported silanisation procedure for immobilising gelatin. *In vitro* cell adhesion and culture studies with human periosteum-derived cells showed that the investigated coatings did not compromise the biocompatible nature of Ti6Al4V porous implants, but no distinct biological differences between the coatings were found.

1. Introduction

In the field of biomedical research, titanium (Ti) and its alloys are currently still the metals of choice for orthopaedic implants. They owe this to their appropriate mechanical properties, excellent corrosion resistance and superior biocompatibility. The advantageous surface properties result from the chemical stability and structure of the surface oxide layer. Despite the benefits of this oxide layer, the bone binding capacity or the bio-activity of Ti is not sufficient to realise a chemical bond between the implant and the bone tissue. Ti and bone tissue are usually separated by a thin (ca. 10 nm), non-mineralised protein layer [1]. The bond associated with osseointegration is attributed to a mechanical interlocking between the Ti surface roughness/pores and the newly formed bone tissue [2]. Nevertheless, surface modification of Ti can improve cell adhesion, implant fixation (decrease of micro-movement) and reduce fibrous tissue formation. In this manner, the osseointegration is thus enhanced and bone repair is accelerated. In fact, a higher degree of osseointegration leads to an improved mechanical stability and a reduced risk of implant loosening. Additionally, in orthopaedics, there is an evolution towards personalised porous implants with complex inner structures to adjust for patient specificity and improve osseointegration [3,4]. The combination of a patient specific implant with a highly controllable inner structure and tailored surface characteristics can eventually reduce hospitalisation time/costs and improve the quality of life of the patients [5].

Over the years, researchers have focussed on finding the most suitable surface characteristics to provoke optimal cell and tissue response in order to obtain such an implant with improved clinical outcomes.

Different methods to improve the initial interaction of the Ti surface with the surrounding bone tissue are being explored worldwide, including the deposition of calcium phosphates [6–10], the adsorption of proteins like collagen [11–14] and fibronectin [15–18], the attachment of peptides by the use of self-assembled monolayers (SAM's) and polymer coatings [19–22]. Binding of molecules to the surface can be physical

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(through adsorption or incorporation into the top surface layer), or chemical (through covalent bonds) [1,23]. In the latter case, most of the strategies are based on the introduction of reactive groups onto the Ti surface, such as by silanisation reactions [24–27].

In a previous study from our research group, gelatin was immobilised onto the Ti surface using a silanisation reaction [28]. Gelatin is a water soluble, biodegradable protein, derived from collagen, one of the main constituents of the extra cellular matrix, by partial hydrolysis (acidic or alkaline) [29]. Due to its cell attractive nature (presence of RGD sequences) gelatin can bio-activate the surface of a Ti implant. Despite the promising results of our previous study, the disadvantages of a silanisation reaction, like the dependence on the number of hydroxyl groups on the Ti surface and the hydrolytical instability of the resulting siloxane layer, drove the search for an alternative immobilisation strategy. In our work, we selected polydopamine as prime layer for the subsequent deposition of gelatin. The idea is based on the composition of mussel adhesive proteins which are known to attach to virtually any surface. Lee *et al.* identified dopamine [2-(3,4-dihydroxyfenyl) ethylamine] as the smallest structural imitation component of these adhesive proteins. Furthermore, dopamine polymerises spontaneously onto any surface (noble metals, oxides, ceramics, polymers and semiconductors) into a thin polydopamine film, which can act as a prime layer for depositing functional coatings [30]. Recent insights into the structure of this polydopamine are still not sufficient to account for its properties. Opposed to the assumed 'open-chain polycatechol/quinine model', on the one hand, and the 'eumelanin model', on the other hand, Dreyer *et al.* suggested that polydopamine is not a covalent polymer but a supramolecular aggregate of monomers [31]. Simultaneously, Hong *et al.* reported on a physical, self-assembled trimer entrapped within polydopamine [32]. Even more recently, Della Vecchia *et al.* revealed a three-component structure of polydopamine. Nevertheless, they suggest representing polydopamine as a collection of oligomeric species in which monomer units are linked through different bonding. The coexistence of such structurally diverse components accounts for a unique blend of eumelanin-like and amine containing polycatechol functionalities [33]. Despite the so far unknown structure of polydopamine, the technique has previously been successfully applied on Ti and Ti alloy surfaces to immobilise biomolecules, such as dextrane, chitosan, hyaluronic acid, heparin, gelatin and growth factors [34–40]. Besides the advantageous characteristics of being a water-based and non-toxic coating strategy, it also presents a straightforward and versatile method to coat a wide variety of surfaces, ranging from that of a 2D substrate to that of complex 3D porous implants. Hence, its potential for future use on implants is secure as these final implants can have very customised morphologies which at this moment is a limitation for the present line of sight coating techniques.

In the present paper we describe the application of a stable gelatin coating onto a Ti6Al4V surface by applying an intermediate polydopamine coating. In an attempt to increase the biofunctionality of the surface even more, a final coating of the cell adhesive protein fibronectin was applied onto the gelatin. Due to the natural binding affinity between fibronectin and gelatin a natural presentation of the immobilised fibronectin on the surface and, hence, a retention of its cell adhesive activity, is anticipated [41]. The coatings were optimised on two dimensional (2D) Ti substrates and characterised via static contact angle (SCA) measurements, X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and fluorescence microscopy.

In the second part of this study, the optimised gelatin coating technology was applied onto three dimensional (3D) porous Ti6Al4V implants (height: 3 mm diameter: 6 mm) which were fabricated via additive manufacturing (AM) technology, i.e. selective laser melting (SLM) [42–44]. 3D micro-computed tomography (Micro-CT) imaging was used to characterise the average pore and strut size, porosity and surface area of the as-produced porous implants. The as-produced Ti6Al4V porous implants were divided into four categories: (i) uncoated or oxidised, (ii) polydopamine coated (Dop), (iii) polydopamine/gelatin coated (Dop-Gel), and (iv) polydopamine/gelatin/fibronectin coated (Dop-Gel-Fn). Transfer of the coating technology from Ti to Ti6Al4V surfaces was straightforward since the surface composition of the oxidised alloy largely compares to the one of the oxidised, pure Ti surface [45,46]. The effects of the different coatings on *in vitro* cell seeding efficiency (CSE), proliferation and differentiation of a clinically relevant mesenchymal stem cell-like osteoprogenitors, namely human periosteum-derived cells (hPDCs), were evaluated.

2. Materials and methods

2.1. Materials

Ti foils (0.125 mm thick, 99.6% pure according to the data sheet) were obtained from Chempur (Karlsruhe, Germany). Cylindrical porous Ti6Al4V implants (6 mm diameter × 3 mm height) were fabricated by an in-house selective laser melting (SLM) machine (Fig. 1) [44,47–49]. The average as-produced pore size, strut size, porosity and surface area, as shown in Fig. 1, were assessed by micro-CT image analysis.

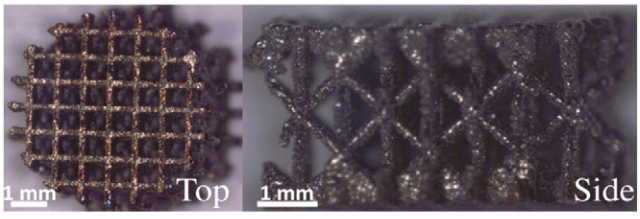
Concentrated hydrochloric acid (HCl; 37%) was provided by Panreac Quimica S.A (Barcelona, Spain), hydrogen peroxide (H_2O_2; 30%) and dimethylformamide (DMF) by Aldrich (Bornem, Belgium) and ammonium hydroxide (NH_4OH; 25%) by Acros (Geel, Belgium). HPLC grade acetone, HPLC grade pentane and 2-(3,4-dihydroxyphenyl)ethylamine hydrochloride (dopamine hydrochloride) were all purchased from Sigma (Bornem, Belgium). Cyclohexane was obtained from Fiers (Kuurne, Belgium), it was dried with calcium hydride and distilled before use. Gelatin type B, isolated from bovine skins after an alkaline pre-treatment, was a kind gift from Rousselot (Ghent, Belgium). Gelatin with an isoelectric point of ca. 5, gel strength of 257 Bloom and viscosity (6.67%, 60 °C) of 4.88 mPa s was used. Oregon Green® 488, carboxylic acid, succinimidyl ester was purchased from N.V. Invitrogen SA (Ghent, Belgium). Fibronectin from bovine plasma (0.1% solution, 1 mg/ml in 0.5 M NaCl, 0.05 M Tris-HCl and pH = 7.5) was obtained from Sigma-Aldrich (Bornem, Belgium). The water used throughout this study was milliQ (double distilled, >18 MΩ/cm).

2.2. Methods

2.2.1. Pre-treatment of Ti and Ti6Al4V

Cleaning of the metal substrates and porous implants consisted of sequenced ultrasonic treatments in (1) cyclohexane (10 min), (2) 10 N HCl (30 min) and (3) milliQ water (30 min). After cleaning, the samples were oxidised for 20 min in a 1/1/5 $NH_4OH/H_2O_2/H_2O$-mixture. These steps are described in more detail in a previous paper [28].

A



B

N=5	
Average pore size (μm)	621(±10)
Average strut size (μm)	217 (±6)
Porosity (%)	80 (±1)
Surface area (mm²)	262 (±6)

Fig. 1. (A) Cylindrical Ti6Al4V porous implants made by selective laser melting (6 mm diameter × 3 mm height). (B) Morphological parameters of the as-produced Ti6Al4V porous implant assessed by micro-CT image analysis. Mean (±standard deviation).